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Immunization of Balb/C mice by Uropathogenic *E.coli* Recombinant PapG.AcmA Protein

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Urinary tract infection (UTI) caused by *Escherichia coli* (UPEC) is one of the most common infections in the world today, which has become resistant to most antibiotics. Despite many efforts, there is no vaccine to protect humans against the infection. PapG.AcmA recombinant protein can be a particular vaccine to treat this infection. Because it helps prevent bacteria from sticking to the surface of the host cell. In this study, after the purification of the protein with Ni-NTA column, the immunity of protein was assessed in Balb/C mice. Experimental mice were divided into three groups and were immunized with Freund adjuvanted PapG.AcmA protein or alum adjuvanted protein or PBS (as control group). Then, lymphocyte proliferation assay was conducted via Brdu/ELISA based methods, IL-4 and IFN-γ cytokines secretion was quantified with commercial ELISA kit; in addition, total antibody, IgG1 and IgG2a subtyping were assayed using ELISA method. The results show that recombinant PapG.AcmA protein was resulted to induction of cellular and humoral immune responses in the mice. This effect of nano-vaccination strategy (with TLR5 agonists) also varies on different parameters of the immune system; in this regard, the cellular immune responses had a higher stimulation level compared with the humoral immune responses.

Key words: Uropathogenic *E.coli*, UTI, PapG.AcmA, expression, immune responses.

Urinary tract infection (UTI) to colonization and invasion of bacteria to the urinary tract tissue shed[1]. UTI is one of the most common infections in humans are influenced by gender and age. In addition to the high cost of treatment of these infections cause high mortality in the spectrum of the society[2]. Almost 90% of UTI infections by *E. coli* strains Uropathogenic(UPEC) is created[1, 2]. Although 95 percent of the original location UTI is a bladder infection, but it can lower their urinary tract, causing pyelonephritis devices have high administrative and subsequent bacteremia, sepsis and sometimes causing death[3]. UPEC has the whole range of general and specific virulence factors. The virulence factors to facilitate the growth and survival of bacteria in the urinary tract due to host[4, 5]. Among these virulence factors, particularly the different types of cell adhesion plays an essential role. UPEC strains pathogenic cycle connection necessary step to begin colonization of host mucosal surfaces that followed in the wake of the invasion of other host[4].

At the bacteria through the connection process type I and type P fimbriae fimbriae especially occurs. Unlike type I fimbriae mainly

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the lower urinary tract infections are caused P-fimbriae, causing infection in the urinary tract is rising. Ascent of the lower urinary tract infection to the upper parts of the urinary tract, kidney problems and create a context for providing acute pyelonephritis[4, 6]. Type P fimbriae is resistant to mannose which is also called fimbriae pap that the term cell is associated with pyelonephritis. This protein has 3 different alleles papGI, papGII, papGIII is often associated with inflammation of the kidneys and pelvis humans[7]. Type P fimbriae structural protein composed of six top cell protein (papG) to attract bacteria into epithelial cells of the kidney is necessary[8, 9]. Current treatment to reduce and limit administrative infections on the use of antibiotics based on today because of increasing antibiotic resistance and side effects of too much of it, the need for vaccines for the treatment of becomes clearer[4]. To date, several UTI vaccine candidate is based on *E. coli* Uropathogenic But none of these reasons is not necessary to stage the human phase of new vaccines against UIC shows[4, 10]. Since Uropathogenic Escherichia coli (UPEC) of different mechanisms to overcome the hosts and their survival is therefore a suitable candidate vaccines based on recombinant antigens for adsorption on the surface of bacterial cells[11, 12]. PapG protein because of immunological stimulation and lack of diversity among the isolates of pathogens and lack of antigenic shift and antigenic similarity is a good candidate for a vaccine[13, 14].

In the present study, the immunity responses against the produced recombinant PapG protein was evaluated in the murine model.

MATERIALS AND METHODS

The PapG.AcmA protein expression

The construction and expression of the PapG.AcmA protein was done in our previous study[15].

Mice

Six-to-eight weeks inbred female BALB/c mice were purchased from Pasteur Institute of Iran (Karaj, Iran). Mice were housed for one week before immunization, given free access to food and water and maintained in 12h light and 12h dark cycles condition accordance with the Animal Care and Use Protocol of Islamic Azad University.

Experimental groups and immunization

Experimental mice (n=18) were divided into 3 groups (n=6). The first group was immunized with 20 µg of PapG.AcmA protein adjuvanted in complete Freund's adjuvant. The second group of mice was immunized with 20 µg of PapG.AcmA protein adjuvanted in alum. The third group of mice was injected with PBS as control group. Immunization carried out subcutaneously, three times with 2 weeks interval.

Lymphocyte proliferation assay

Two weeks after last shooting of vaccine, the spleens of mice were re-suspended in cold PBS containing 5% FBS. RBCs were lysed with lysis buffer and after centrifugation, the pellet was re-suspended and adjusted to 3×10^6 cells/ml in RPMI 1640 (Gibco, Germany) supplemented with 10% FBS, 4 mM/L-glutamine, 1mM sodium pyruvate, 50µm 2ME, 100µg/ml streptomycin and 100 IU/ml penicillin. Then 100 µl of the cell suspension was dispensed into 96-well flat-bottom culture plates and stimulated with 10 µg/ml of PapG.AcmA protein. Phytohemagglutinin-A (5 µg/ml, Gibco) was used as a positive control, un-stimulated wells were used as the negative controls and complete culture medium was used as blank. Experiments were done in triplicate and after three days of culture, 100 µl of 5-bromo-2-deoxy-uridine labeling solution was added into each well and incubation continued for 18 hrs. Next, the plates were centrifuged and culture medium was removed completely and dried at 60 ° C for 30 minutes. Subsequently, 100 µl of anti-Brdu antibody was added to each well for 2 hrs and the plates were washed five times with PBS and 100 µl of TMB substrate was added to each well in dark condition. The reaction was stopped by adding 100 µl of 2N H_2SO_4. Absorbance in each well was measured using a spectrophotometric plate reader at 450/630 nm. Stimulation Index (SI) was calculated according to the formula: OD of stimulated wells/OD of un-stimulated wells.

IFN-γ and IL-4 Cytokines ELISA

Two weeks after final immunization, a total number of 3×10^6 splenocytes were cultured on each well of 24-well plate in complete RPMI-1640 medium and stimulated with 10 µg/ml of PapG.AcmA protein and incubated at 37°C in 5% CO_2. Three days later, supernatants were collected and IFN-ã

and IL-4 cytokines were assessed via commercial ELISA Kits (Mabtech, Sweden) according to the manufacturer's instructions. The quantity of cytokines was reported as pg/ml according to related standard curve.

ELISA of total antibodies and IgG1, IgG2a subclasses

Two weeks after last injection, sera of mice were collected and total antibodies were determined by an optimized ELISA method. Briefly, 100 µl of 10 µg/ml of PapG.AcmA antigen in PBS buffer were added into 96-well ELISA Maxisorp plates (Nunc, Naperville, IL) and incubated overnight at 4 °C. The wells washed three times with PBS containing 0.05% Tween 20 (washing buffer) and blocked 1 h at 37 °C with 5% skimmed milk in PBS (blocking buffer). Plates were washed with washing buffer and 100µl of 1/50 to 1/6400 dilutions of sera were added to each wells and incubated at 37 °C for 2 hrs. The wells washed five times with washing buffer and incubated for 2 h with 100 µl of 1/10000 dilution of anti-mouse HRP conjugate (Sigma, USA). The wells washed six times and incubated 30 min with 100 µl of TMB substrate in the dark and reaction was stopped with adding 100 µl of 2N H2SO4 and color density was measured at A_{450}nm versus A_{630}nm as references with ELISA plate reader. Also, specific IgG1and IgG2a subclasses were assessed with goat anti mouse IgG1and IgG2a secondary antibodies (Sigma, USA) according to the manufacture's protocol.

Statistical analysis

All experiments were performed in triplicate and the data was expressed as means ± S.D of each experiment. All statistical analyses carried out by T-Test using one way annova test using SPSS v18 software. In all of the cases, *P* values < 0.05 were considered to be statistically significant.

RESULTS

Lymphocyte proliferation

Results of lymphocyte proliferation indicates that PapG.AcmA protein adjuvanted with alum increased lymphocyte proliferation as compared to control group (p=0.0001). Also immunization of mice with PapG.AcmA/alum increased lymphocyte proliferation in comparison to PapG.AcmA/freund immunized group (p=0.0011). Furthermore, immunization of mice with PapG.AcmA formulated with freund adjuvant increased lymphocyte proliferation compared to the control group (p= 0.0708) (fig.1).

IL-4 and IFN-ã cytokine assay

Result of IL-4 cytokine analysis showed that immunization with candidate vaccine formulated with freund adjuvant increased IL-4 cytokine compared to the control group (p= 0.0182). Also, immunization with candidate vaccine adjuvanted with alum increased IL-4 cytokine compared to control group (p= 0.1282). There is no significant difference between alum and freund adjuvanted groups in the induction of IL-4 cytokine.

Assessment of IFN-ã cytokine in the experimental groups show that, immunization with PapG.AcmA/ freund increased IFN-ã cytokine as compared to the control group (p=0.0008). Also immunization with PapG.AcmA/Alum increased IFN-ã cytokine level in comparison to the control group (p=0.005). Analysis of IFN-ã cytokine release in PapG.AcmA/ freund group in comparison to the PapG.AcmA/Alum group show no significant difference (p=0.7069).

Total antibody

Results of total antibodies assessment in the experimental groups indicated that immunization with the PapG.AcmA/ alum increased the total IgG responses compared to control group at dilutions of 50,100 and 200 (p<0.0394). Also vaccine candidate adjuvanted in freund adjuvant increased the total antibodies responses compared to control group at dilutions of 50, 100, 200, 400 and 800 (p<0.0188). Significant differences was not observed between ferund and alum adjuvanted groups in the induction of total IgG response.

IgG1 and IgG2a isotypes

Evaluation of specific IgG1 isotype in the experimental groups show that immunization with PapG.AcmA/ alum and PapG.AcmA/ freund increased IgG1 level in comparison to the control group (p< 0.0001). Immunization with PapG.AcmA/ alum increased IgG1 level in comparison to the PapG.AcmA/ freund but was not significantly difference (p= 0.9838) (figure 1).

IgG2a isotype responses showed that immunization with PapG.AcmA/alum and also PapG.AcmA/ freund increased IgG2a level compared to control group (p= 0.0374). No

significant difference was observed in the IgG2a level between PapG.AcmA/alum and PapG.AcmA/ freund immunized groups (p= 0.8604) (figure 1).

DISCUSSION

The vaccine utilization is the most safety and economic tools for inhibition and even therapy of the infectious diseases[12]. The *E. coli* strains are the most important cause of urinary tract infection in human[1-3]. Furthermore, the use of antibiotic treatment was faced to antibiotic resistant origination. Therefore, a vaccine is the best fighting choice against UTI[3, 13, 16]. The UPEC attachment to the bladder is an important and essential step in the colonization and pathogenesis of the disease. In this regard, targeting the attachment and abrogation step can be resulted in the control and prevention of the infection. Currently, vaccine strategies versus UTI focused on the inhibition of bacterial binding molecules as targets[16-18].

Pili type P of UPEC is one of the essential agents of the urinary tract infection[9, 11, 12]. According to the various reports, the binding process in uropathogenic *Escherichia coli* is

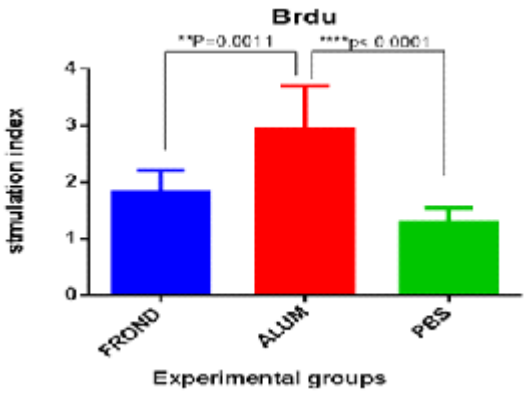

Fig. 1. The lymphocyte proliferation results in immunized mice with formulated freund/ alum adjuvanted PapG.AcmA and PBS

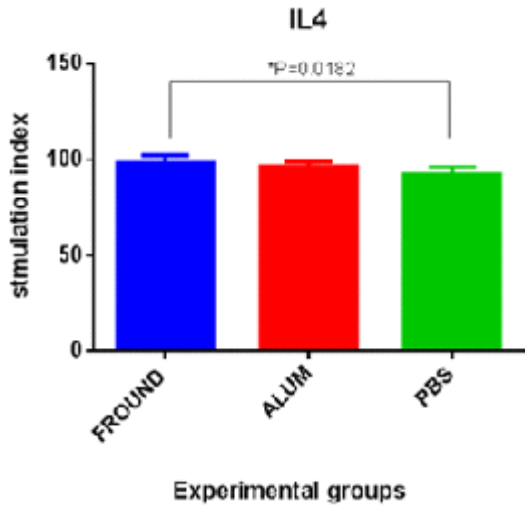

Fig. 2. The IL-4 cytokine levels of the immunized mice in different groups

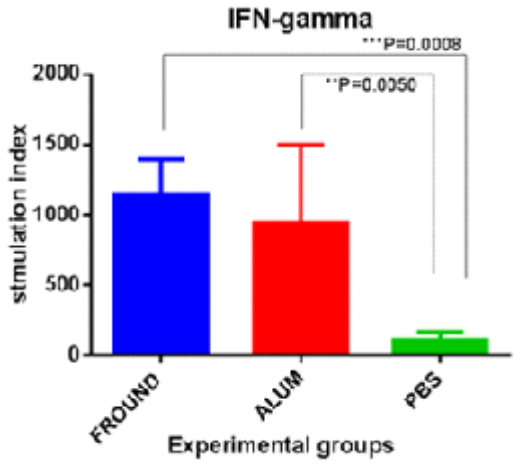

Fig. 3. The IFN-γ cytokine levels of the immunized mice in different groups

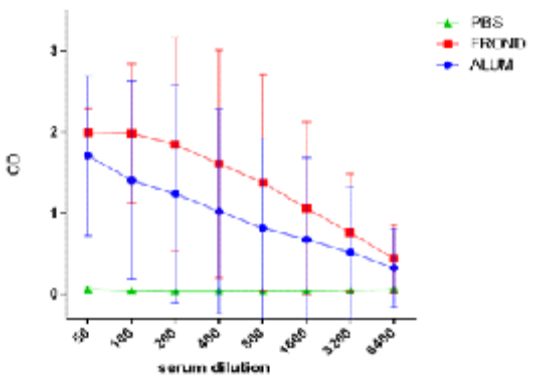

Fig. 4. The results of total antibodies assessment in different groups

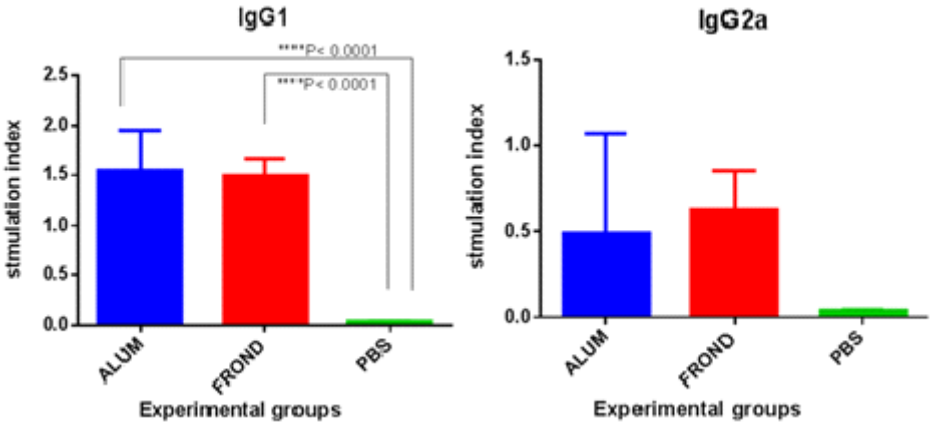

Fig. 5. The results of IgG1 and IgG2a isotypic antibodies assessment in different groups

facilitated through type 1 and P fimbriae; P fimbria causes the ascending infections in the urinary tract. Ascending of the urinary tract infection from the lower parts to the upper urinary tract system provides the progression of infection to the kidney, establishing pyelonephritis [2, 9]. Considering the mechanism of pyelonephritis establishment and the critical role of PapG in the formation of pyelonephritis, it can be considered as a suitable vaccine candidate for UTI. In this study, the recombinant PapG.AcmA protein was expressed in *E.coli*. Herein, the PapG.AcmA gene segment was cloned into the PET21a expressing vector and then expressed in *E. coli* strain origami, as a host. In the following, the recombinant protein PapG.AcmA was purified and used as a vaccine candidate in immunoassay using alum as well as freund's adjuvants. Results of lymphocyte proliferation showed that immunization of mice with PapG.AcmA/alum as well as freund's adjuvants increase lymphocyte proliferation when compared to the control group. Lymphocyte proliferation as a cellular immunity marker[19, 20] showed the ability of PapG.AcmA protein in the induction of cellular immunity.

Several studies showed that cellular immune responses play important roles in the clearance of infection in UTI[21-24] and PapG.AcmA, as a vaccine candidate, can successfully induce cellular immune responses. Cytokine analysis showed that PapG.AcmA, as a vaccine, in combination with both alum and freund's adjuvants could strongly induce in immune-deficient IFN-g secretion and polarize the Th1 pattern. A study of Carson *et al*. mice showed that the Th1 immune platform and cellular immunity play important roles in the resistance to UTI[23]. The results of cytokine assay confirmed the ability of PapG.AcmA protein to induce Th1 cytokine profile, and the possible potency of this vaccine to induce the resistance to the infection. Considering the involvement of antibodies in the resistance to UTI, total antibodies were then evaluated. The results of total antibodies showed that immunization with PapG.AcmA/alum and freund's adjuvants significantly increased total antibodies as compared to the control group. Various studies showed the role of humoral immune responses in the resistance to the infection[22, 25, 26]. In fact, antibodies masking the adhesion molecules on UPEC are able to inhibit the attachment to the host cells and abrogate colonization and subsequently the infection[26] and PapG.AcmA, as an adhesion molecule, can induce strong antibody responses with increased IgG1 and IgG2a classes. Taken together, in the preset study we have shown the potency of the PapG.AcmA molecule as an immunogen in the induction of humoral and cellular immune responses. However, in the near future, the PapG vaccine using the surface display strategy would be evaluated in the experimental UTI challenges.

CONCLUSION

The results show that the recombinant PapG.AcmA protein induces the cellular and

humoral immune responses in Balb/C mice. This effect of nano-vaccination strategy (with TLR5 agonists) also varies on different parameters of the immune system; in this regard, the cellular immune responses had a higher stimulation level compared with the humoral immune responses.

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